Hemolytic Steroid Disulfates from the Far Eastern Starfish *Pteraster pulvillus*

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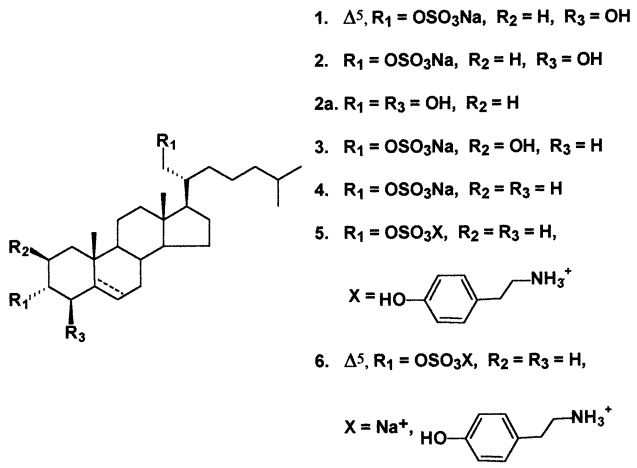
Six steroidal ophiuroid-type disulfates, namely, disodium salts of (20*R*)-cholest-5-ene-3α,4*β*,21-triol 3,21-disulfate (**1**), (20*R*)-5α-cholestane-3α,4*β*,21-triol 3,21-disulfate (**2**), (20*R*)-5α-cholestane-2*β*,3α,21-triol 3,21-disulfate (**3**), and (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate (**4**), the dityrammonium salt of (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate (**5**), and a mixture of sodium and tyrammonium salts (1:1) of (20*R*)-cholest-5-ene-3α,21-diol 3,21-disulfate (**6**), have been isolated from the ethanolic extracts of Far Eastern starfish *Pteraster pulvillus*. Steroids **2** and **3** are new compounds. Steroids **1**, **2**, and **4**−**6** showed hemolytic activity to mouse erythrocytes with HC_{50} values of 8.0×10^{-5}, 4.5×10^{-5}, 1.0×10^{-5}, 1.8×10^{-5}, and 3.3×10^{-5} M, respectively.

Marine organisms contain a great number of sulfated steroids. Among them, disulfates attract considerable interest because of their diverse structures and biological activities. These compounds have been found in sponges, starfish, and especially in ophiuroids, in which they may be considered to be the most characteristic secondary metabolites.[1−3] Steroidal disulfates from ophiuroids differ from other marine disulfates in some of their structural features, such as the presence of sulfate groups at the 3α and 21 positions. Quite recently, it was shown by Levina et al.[4,5] that some starfish belonging to the genus *Pteraster* contained ophiuroid-type 3,21-disulfates instead of polyhydroxylated steroids and related mono- and biosides, which are more characteristic for the Asteroidea class. Taking into account that representatives of the Pterasteridae family have some unusual features of reproduction and that this taxon probably combines a group of living fossils, this fact is interesting from the perspective of phylogenetic relationships between the Asteroidea and Ophiuroidea classes. That is why we study the steroidal composition of any species belonging to this family with great attention.

Continuing our studies of polar steroidal constituents from the Far Eastern starfish,[6,7] we have analyzed the extracts of the starfish *Pteraster pulvillus* M. Sara (order Velatida, family Pterasteridae) collected by trawling at a depth of 110 m in the Sea of Okhotsk. We have obtained steroidal ophiuroid-type disulfates (**1**−**6**), two of which, the disodium salts of (20*R*)-5α-cholestane-3α,4*β*,21-triol 3,21-disulfate (**2**) and (20*R*)-5α-cholestane-2*β*,3α,21-triol 3,21-disulfate (**3**), are new natural products. Chromatography of the EtOH extract of this starfish on Amberlite XAD-2, Sephadex LH-20, Si gel, and Florisil followed by reversed-phase HPLC on a Zorbax ODS and Nucleosil C_{18} columns was employed.

Steroid **1** showed a pseudomolecular cation peak at *m*/*z* 645 $[M + Na]^+$ in the positive MALDI-TOF mass spectrum and peaks at *m*/*z* 599 $[M - Na]^-$ and 577 $[M - 2Na + H]^-$ in the negative MALDI-TOF mass spectrum. The NMR spectra of **1** were identical with those of (20*R*)-cholest-5-ene-3α,4*β*,21-triol 3,21-disulfate from the ophiuroids *Ophiotrix fragilis*, *Ophiura texturata*, and *Ophionotus victoriae*.[8] On this basis, **1** was identified as the disodium salt of (20*R*)-cholest-5-ene-3α,4*β*,21-triol 3,21-disulfate. Besides ophiuroids, this compound was recently isolated from the starfish *Pteraster tesselatus* and *Pteraster* sp.[5]



The pseudomolecular ion of **2** at *m*/*z* 647.2254 $[M + Na]^+$ in the positive high-resolution MALDI-TOF mass spectrum as well as the ^{13}C and ^1H NMR data indicated that the molecular formula was $C_{27}H_{46}O_9S_2Na_2$. The negative MALDI-TOF mass spectrum of **2** showed peaks at *m*/*z* 601 $[M - Na]^-$ and 579 $[M - 2Na + H]^-$. The ^{13}C and DEPT NMR spectra exhibited the presence of 27 carbon atoms in the molecule, including four methyl groups, 12 methylenes, nine methines, and two quaternary carbons, indicating that this steroid also possessed the cholestane skeleton. There were ^{13}C NMR signals observed at *δ* 69.4, 74.1, and 78.6 corresponding to three carbon atoms bound to oxygen. $^1H-^1H$ COSY and HMQC experiments allowed us to assign the signals of all protons and carbons in NMR spectra (Table 1). Two proton signals at *δ* 3.90 (dd, *J* = 6.9, 9.6 Hz) and 4.18 (dd, *J* = 3.8, 9.6 Hz) in the ^1H NMR spectrum of **2** are characteristic of a CH_2-21 group connected to an *O*-sulfate group. Chemical shifts of the carbons of C-11−C-18 and C-20−C-27 in ^{13}C NMR spectrum of compound **2** coincided with those of the previously described 5α-cholestane-3α,21-diol 3,21-disulfate.[9] On this basis we concluded that two other hydroxyl groups were situated in rings A and B. The multiplicities and small coupling constants of the two methine proton signals at *δ* 4.39 (q, *J* = 2.8 Hz) and 3.76 (t, *J* = 2.8 Hz) corresponded to equatorial positions for protons H-3 and H-4 and, therefore, a geminal 3α,4*β*-

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Table 1. ^{1}H and ^{13}C NMR Data (CD_3OD) for Compounds **2** and **3**[a]

	2		**3**	
carbon	δ_C mult[b]	δ_H mult	δ_C	δ_H mult
1	33.6 t	1.30 m, 1.44 m	40.6	1.35 m, 1.77 m
2	23.4 t	1.83 m (e), 1.99 m (a)	70.1	4.06 brd (2.5)
3	78.6 d	4.39 q (2.8)	78.5	4.39 brd (2.5)
4	74.1 d	3.76 t (2.8)	30.3	1.57 m (e), 1.82 m (a)
5	45.4 d	1.42 m	40.8	
6	26.5 t	1.26 m, 1.72 m	29.3	
7	33.6 t	0.96 m, 1.76 m	33.2	
8	36.9 d	1.39 m	36.4	
9	56.7 d	0.69 m	56.6	
10	36.6 s		36.4	
11	21.3 t	1.27 m, 1.48 m	22.0	
12	40.4 t	1.20 m, 1.98 m	41.1	
13	43.5 s		43.6	
14	57.9 d	1.05 m	57.8	
15	25.1 t	1.06 m, 1.61 m	25.1	
16	28.6 t	1.36 m, 1.84 m	28.7	
17	51.9 d	1.43 m	52.0	
18	12.7 q	0.71 s	12.7	0.72 s
19	14.8 q	1.02 s	14.6	0.99 s
20	41.4 d	1.66 m	41.4	1.65 m
21	69.4 t	3.90 dd (6.9, 9.6), 4.18 dd (3.8, 9.6)	69.5	3.91 dd (6.5, 9.5), 4.18 dd (3.6, 9.5)
22	30.8 t	1.30 m, 1.44 m	30.9	
23	24.4 t	1.29 m, 1.44 m	24.3	
24	40.7 t	1.15 m (2H)	40.6	
25	29.1 d	1.56 m	29.1	1.55 m
26	23.0 q	0.88 d (6.6)	23.0	0.88 d (6.6)
27	23.2 q	0.88 d (6.6)	23.2	0.88 d (6.6)

[a] J (Hz) values are shown in parentheses. Assignments from 300 MHz ^{1}H−^{1}H COSY and HMQC data. [b] Multiplicity by DEPT.

position of hydroxyl groups. The comparison of the chemical shifts of carbons C-3 (δ 78.6) and C-4 (δ 74.1) with those of the 5α-cholestane-3α,4β-diol (C-3 δ 70.3; C-4 δ 76.1) indicated the position of the second sulfate group was at C-3.[10] The stereochemistry at C-20 in **2** was determined to be 20*R* in accordance with the chemical shifts and the shapes of the CH_2-21 signals in the ^{1}H NMR spectrum of **2a** (δ 3.70, m, $W_{1/2}$ = 11 Hz).[9] Therefore, the structure of **2** was established as the disodium salt of (20*R*)-5α-cholestane-3α,4β,21-triol 3,21-disulfate. The 3α,4β-dihydroxy substitution of the saturated 5α-cholestane skeleton system is now described for the first time in steroids from starfish or ophiuroids.

The molecular formula of compound **3** was deduced to be $C_{27}H_{46}O_9S_2Na_2$ on the basis of a pseudomolecular ion at *m/z* 647.2297 $[M + Na]^+$ in the high-resolution positive MALDI-TOF mass spectrum and the NMR data. In the negative MALDI-TOF mass spectrum of **3** we observed anion peaks at *m/z* 601 $[M - Na]^-$ and 579 $[M - 2Na + H]^-$. The ^{13}C NMR spectrum (Table 1) showed that **3** contained 27 carbon atoms, including three signals of carbon atoms bound to oxygen at δ 69.5, 70.1, and 78.5 ppm. The NMR data for the side chain of **3** (Table 1) closely resembled those of **1** and **2**, indicating the presence of a saturated cholestane-type side chain with a sulfate group at C-21. Because the chemical shifts and coupling constants of the two methine protons at δ 4.06 (brd, J = 2.5 Hz) and 4.39 (brd, J = 2.5 Hz) in the ^{1}H NMR spectrum of **3** were very similar to those found in (20*R*)-5α-cholest-24-ene-2β,3α,21-triol 3,21-disulfate and (20*R*)-5α-cholest-24-ene-2β,3α,21-triol 2,21-disulfate,[11] the position of the sulfate group in the steroidal nucleus of **3** was determined by the ^{13}C NMR data. According to data found in the literature,[11] the chemical shifts of C-1 at δ 38.9 ppm and C-4 at δ 32.5 ppm in the ^{13}C NMR spectrum of 2β,3α-diol are characteristic of the presence of an *O*-sulfate group at C-2, while the chemical shifts of C-1 at δ 40.5 ppm and C-4 at δ 30.3 ppm in the ^{13}C NMR spectrum of the same steroid diol indicated the position of an *O*-sulfate group at C-3. The coincidence of the carbon signals from the steroidal nucleus of **3** and (20*R*)-5α-cholest-24-ene-2β,3α,21-triol 3,21-disulfate and, in particular, chemical shifts of the signals of C-1 (δ 40.6) and C-4 (δ 30.3) demonstrated that compound **3** possessed a 2β,3α-diol 3-sulfate steroidal pattern. ^{1}H−^{1}H COSY and HMQC experiments allowed for the assignment of characteristic protons in the ^{1}H NMR spectrum of this compound. Thus, the structure of **3** was established as the disodium salt of (20*R*)-5α-cholestane-2β,3α,21-triol 3,21-disulfate.

The positive MALDI-TOF mass spectrum of **4** exhibited a pseudomolecular cation at *m/z* 631 $[M + Na]^+$, and the negative MALDI-TOF mass spectrum showed anions at *m/z* 585 $[M - Na]^-$ and 563 $[M - 2Na + H]^-$. These data indicated the presence of two sulfate groups in **4**. In the ^{13}C NMR spectrum of **4** the signals of 27 carbon atoms were observed including those of oxygen-bearing atoms at δ 69.4 and 76.7. The NMR data of **4** were identical with those reported by Riccio et al.[9] for (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate from the ophiuroid *Ophioderma longicaudum*. On this basis **4** was identified as the disodium salt of (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate.

The negative MALDI-TOF mass spectrum of **5** showed ions at *m/z* 585 $[M - 2 \text{ cations} + Na]^-$ and 563 $[M - 2 \text{ cations} + H]^-$. In addition to the signals from the steroid moiety, which were consistent with (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate, commonly found in ophiuroids, ^{1}H and ^{13}C NMR spectra of **5** also contained signals corresponding to tyramine.[7,12] For instance, the ^{1}H NMR spectrum contained two aromatic doublets at δ 7.08 (2H, J = 8.7 Hz) and 6.76 (2H, J = 8.7 Hz) and two methylene triplets at δ 3.10 (2H, t, J = 7.6 Hz) and 2.84 (2H, t, J = 7.6 Hz), and the ^{13}C NMR spectrum contained carbon signals at δ 157.7 (C-1′), 130.8 (C-2′, C-6′), 128.5 (C-4′), 116.9 (C-3′, C-5′), 42.8 (CH_2−N<), and 33.8 (Ar−CH_2). The positive MALDI-TOF mass spectrum of compound **5** showed a tyrammonium cation at *m/z* 138. The comparison of the proton signal intensities for the tyramine and the steroidal moieties in the ^{1}H NMR spectrum showed that **5** was a dityrammonium salt of (20*R*)-5α-cholestane-3α,21-diol 3,21-disulfate. Steroidal sulfates usually only occur in starfish and ophiuroids as sodium salts. Only two cases have been reported in which sulfated steroids were isolated from starfish as tyrammonium salts.[7,12]

In the positive MALDI-TOF mass spectrum of **6** the pseudomolecular cation peak at *m/z* 629 $[M + Na]^+$ and the tyrammonium cation with *m/z* 138 were observed. The negative MALDI-TOF mass spectrum of **6** exhibited anions at *m/z* 583 $[M - 2 \text{ cations} + Na]^-$ and 561 $[M - 2 \text{ cations} + H]^-$. The ^{1}H and ^{13}C NMR spectra of **6** differ from those of **5** mainly in the intensities of some of the signals and in the presence of a 5(6)-double bond. Moreover, the chemical shifts and the coupling constants of the protons of the steroidal part of **6** were identical to those of (20*R*)-cholest-5-ene-3α,21-diol 3,21-disulfate from *Ophioderma longicaudum*.[9] The chemical shifts of the carbon atoms of the steroidal part of **6** coincided with those of the same compound from *Ophiura sarsi* and *Stegophiura brachiactis*.[14] Comparison of the intensities of the tyramine signals and the steroidal moiety in the ^{1}H NMR spectrum of **6** showed that this steroid was a 1:1 mixture of the tyrammonium and the sodium salts of (20*R*)-cholest-5-ene-3α,21-diol 3,21-disulfate.

When tested for hemolytic activity using suspensions of mouse erythrocytes, compounds **1**, **2**, and **4**−**6** showed activity, with HC_{50} values of 8×10^{-5}, 4.5×10^{-5}, 1×10^{-5}, 1.8×10^{-5}, and 3.3×10^{-5} M, respectively.

Recently, the studies on the starfish *Diplopteraster multiples*, belonging to the same family Pterasteridae, was carried out and revealed the presence of ophiuroid-type steroid disulfates in this animal.[14] Thereby, our investigation of the polar steroids from the Far Eastern starfish *Pteraster pulvillus* showed that this species, as well as three other species of the Pterasteridae family examined in our laboratory earlier,[4,5,14] contained characteristic ophiuroid steroidal disulfates. Another species of starfish, *Euretaster insignis*, belonging to the same family and studied by an Italian group, was previously found to contain related steroidal 3*β*,21-disulfates.[15]

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 141 polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-250 spectrometer at 250 and 62.9 MHz, respectively, and on a Bruker DPX 300 spectrometer at 300 and 75 MHz, respectively, using tetramethylsilane as the internal standard. MALDI-TOF mass spectra were recorded on a Bruker Biflex III laser-desorption mass spectrometer coupled with delayed extraction using a N_2 laser (337 nm). Mass spectral samples were dissolved in MeOH (1 mg/mL), and 1 μL aliquots were analyzed using α-cyano-4-hydroxycinnamic acid (CCA) matrix. HPLC separations were conducted on Zorbax ODS (13 μm, 250 × 9.4 mm) and Nucleosil C_{18} (5 μm, 250 × 4.6 mm) columns using a DuPont 8800 chromatograph equipped with a differential refractometer.

Low-pressure column liquid chromatography was performed using Polychrom-1 (Biolar, Latvia), Sephadex LH-20 (Sigma, Chemical Co), Si gel L (40/100 μm, Chemapol, Praha, Czech Republic), and Florisil (100−200 mesh, Koch-Light Laboratories Ltd., U.K.). Si gel plates (4.5 × 6.0 cm, 5−17 μm, Sorbfil, Russia) were used for thin-layer chromatography.

Animal Material. Specimens of *Pteraster pulvillus* were collected by dredging in August 1999 at a depth of 110 m near Kunashir Island in the Sea of Okhotsk (research vessel *Akademik Oparin*, 23th scientific cruise). Species identification was carried out by Dr. S. Sh. Dautov (Institute of Marine Biology, Far East Branch of the Russian Academy of Science, Vladivostok, Russia). A voucher specimen [no. 023-42(2)] is on deposit at the marine specimen collection of the Pacific Institute of Bioorganic Chemistry, Vladivostok, Russia.

Extraction and Isolation. The fresh animals (215 g) were chopped and extracted twice with 70% EtOH at 20 °C. Lipids were removed from the supernatant by extraction with benzene (1 mL per 3 mL of the supernatant). The water/ethanol layer was evaporated, and the residue was dissolved in H_2O (1 L). The H_2O-soluble fraction was passed through a Polychrom-1 column (7.5 × 18 cm) and eluted with distilled H_2O until a negative chloride ion reaction was obtained, followed by elution with 50% EtOH. The combined EtOH eluate was evaporated to give a brownish material (7.3 g), which was chromatographed on a Sephadex LH-20 column (3 × 75 cm) with EtOH/H_2O (2:1). The subfractions containing mixtures of steroidal sulfate were purified by chromatography on a Si gel column (3.7 × 20 cm) using $CHCl_3$/EtOH (8:1 → 1:4). Fractions were analyzed by TLC on Si gel using the eluent system BuOH/EtOH/H_2O (4:1:2) and detected by spraying with H_2SO_4.

HPLC of these fractions on a Zorbax ODS column (13 μm, 250 × 9.4 mm, 3 mL/min) with EtOH/H_2O (65:35) as the eluent system yielded pure **4** (31 mg, R_f 0.58), **5** (27 mg, R_f 0.58), **6** (9 mg, R_f 0.57), and subfractions containing **1**−**3**. Repeated HPLC of the subfractions on a Zorbax ODS column (13 μm, 250 × 9.4 mm, 3 mL/min) with EtOH/H_2O (55:45) as the eluent system and on a Nucleosil C_{18} column (5 μm, 250 × 4.6 mm, 0.5 mL/min) with EtOH/H_2O (45:55) as the eluent system yielded pure **1** (13 mg, R_f 0.55), **2** (12 mg, R_f 0.56), and **3** (0.5 mg, R_f 0.56).

(20*R*)-Cholest-5-ene-3α,4*β*,21-triol 3,21-disulfate, disodium salt (1): amorphous powder; $[\alpha]_D$ −15.7° (*c* 0.35; MeOH); ^{1}H and ^{13}C NMR data were identical with those reported by D'Auria et al.;[8] MALDI-TOF(+) *m/z* 645 $[M + Na]^+$; MALDI-TOF(−) *m/z* 599 $[M - Na]^-$, 577 $[M - 2Na + H]^-$.

(20*R*)-5α-Cholestane-3α,4*β*,21-triol 3,21- disulfate, disodium salt (2): $C_{27}H_{46}O_9S_2Na_2$; amorphous powder; $[\alpha]_D$ +6.5° (*c* 1.1; MeOH); ^{1}H and ^{13}C NMR data, see Table 1; HR MALDI-TOF(+) *m/z* 647.2254 $[M + Na]^+$ (calcd for $C_{27}H_{46}O_9S_2Na_3$, 647.2275); MALDI-TOF(−) *m/z* 601 $[M - Na]^-$, 579 $[M - 2Na + H]^-$.

Solvolysis of 2. A solution of compound **2** (1.5 mg) in aqueous 2 N CF_3COOH (2 mL) was heated at 100 °C for 2 h in a sealed ampule. The reaction mixture was evaporated in vacuo and chromatographed on a Florisil column (1.5 × 3 cm) using hexane/ethyl acetate (6:1 → 1:1) as eluent to give the derivative **2a** (0.5 mg).

(20*R*)-5α-Cholestane-3α,4*β*,21-triol (2a): amorphous powder; ^{1}H NMR (CD_3OD) δ 0.67 (3H, s, H_3-18), 0.87 (6H, d, J = 6.6 Hz, H_3-26,27), 1.02 (3H, s, H_3-19), 3.60 (1H, t, J = 2.8 Hz, H-4), 3.70 (2H, m, $W_{1/2}$ = 11 Hz, H_2-21), 3.89 (1H, q, J = 2.8 Hz, H-3).

(20*R*)-5α-Cholestane-2*β*,3α,21-triol 3,21-disulfate, disodium salt (3): $C_{27}H_{46}O_9S_2Na_2$; amorphous powder; $[\alpha]_D$ +14.0° (*c* 0.05; MeOH); ^{1}H and ^{13}C NMR data, see Table 1; HR MALDI-TOF(+) *m/z* 647.2297 $[M + Na]^+$ (calcd for $C_{27}H_{46}O_9S_2Na_3$, 647.2275); MALDI-TOF(−) *m/z* 601 $[M - Na]^-$, 579 $[M - 2Na + H]^-$.

(20*R*)-5α-Cholestane-3α,21-diol 3,21-disulfate, disodium salt (4): amorphous powder; $[\alpha]_D$ +15.6° (*c* 0.7; MeOH); ^{1}H and ^{13}C NMR data were identical with those reported by Riccio et al.;[9] MALDI-TOF(+) *m/z* 631 $[M + Na]^+$; MALDI-TOF(−) *m/z* 585 $[M - Na]^-$, 563 $[M - 2Na + H]^-$.

(20*R*)-5α-Cholestane-3α,21-diol 3,21-disulfate, dityrammonium salt (5): amorphous powder; $[\alpha]_D$ +5.9° (*c* 0.8; MeOH); ^{1}H and ^{13}C NMR data of steroidal part were identical with those reported by Riccio et al.;[9] ^{1}H and ^{13}C NMR data of tyramine were identical with those for tyrammonium cation;[7,12] MALDI-TOF(−) *m/z* 585 [M − 2 cations + Na]$^-$, 563 [M − 2 cations + H]$^-$.

(20*R*)-Cholest-5-ene-3α,21-diol 3,21-disulfate, mixture of tyrammonium and sodium salts (1:1) (6): amorphous powder; $[\alpha]_D$ −1.2° (*c* 0.5; MeOH); ^{1}H NMR data of steroidal part were identical with those reported by Riccio et al.;[9] ^{13}C NMR data of steroidal part were identical with those reported by Fedorov et al.;[13] ^{1}H and ^{13}C NMR data of tyramine were identical with those for tyrammonium cation;[7,12] MALDI-TOF(+) *m/z* 629 $[M + Na]^+$, 138 $[C_8H_{12}ON]^+$; MALDI-TOF(−) *m/z* 583 [M − 2 cations + Na]$^-$, 561 [M − 2 cations + H]$^-$.

Bioassay. Hemolytic activity was determined as previously reported.[16]

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